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Toxin-Producing Species of *Penicillium* and the Development of Mycotoxins in Must and Homemade Wine

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ABSTRACT A number of penicillium strains belonging to the species *Penicillium roqueforti, P. crustosum, P. paneum* Frisvad, and *P. chrysogenum* were analyzed for their ability to produce the mycotoxins isofumigaclavine A, isofumigaclavine B, festuclavine, roquefortine C, and PR toxin when cultured on three different media. Some of the strongest mycotoxin-producing strains were later inoculated into samples of must (grape juice) before and after wine fermentation. After incubation at 25°C for 1 and 2 weeks it was found that all except one of the penicillium strains were able to produce one or more of the toxins analyzed. However, the types of toxins as well as toxin concentrations varied a great deal, depending on culturing medium or culturing time. The media containing yeast extract normally gave higher toxin levels. From the wine experiments it was shown that isofumigaclavine A can be formed under certain circumstances in must and wine. A qualitative High Performance Liquid Chromatography (HPLC) method for simultaneous determination of isofumigaclavines A and B, roquefortine C, and PR toxin was also developed. *Nat. Toxins 5:86–89, 1997.* © 1997 Wiley-Liss, Inc.

Key Words: *Penicillium roqueforti; Penicillium crustosum;* neurotoxins; wine

INTRODUCTION

Two cases of acute poisoning after consumption of homemade wine prepared from apples/rhubarbs and plums, respectively, were reported to the Swedish National Food Administration some years ago. The described effects of the poisoning were trembling, headache, palpitating heart, general weakness, and unconsciousness. In one of these wines, a detected contamination by *Penicillium roqueforti* was suspected to be the cause of the poisoning. In a case reported by Cole et al. [1983], a man suffered similar acute effects from a beer which probably was contaminated with *Penicillium crustosum.* Both *P. crustosum* and *P. roqueforti* are producers of several neurotoxins such as roquefortine C, PR toxin, isofumigaclavine A, isofumigaclavine B, and festuclavine. The toxicological effects of these substances are not well-known, but some neurotoxic data are described by Ohmomo et al. [1975], Scott [1984], and Scott et al. [1976]. In this study 14 penicillium strains were analyzed for their ability to produce neurotoxins. Some strains were also used in experiments to find out if neurotoxin-producing mold strains could grow and produce toxins before, during, and after a yeast fermentation process.

MATERIALS AND METHODS

Strains and Methods

Nine isolates of *P. roqueforti,* three isolates of *P. crustosum,* and one each of *P. paneum* Frisvad and *P. chrysogenum* were tested for their ability to produce toxins in three different media. Of the 14 penicillium strains used in the study, eight came from our own collection and six were obtained as a gift from Ulf Thrane at the Technical University, Copenhagen in Denmark. The strains are given in Table I, where the Danish strains are marked with an Institute for Bio Technology (IBT) number. All strains were checked for purity by culturing on malt extract agar according to the method of Blakeslee [Samson et al., 1995]. The strains were then subcultured to agar slants in tubes containing three different media, namely, Czapek yeast extract agar (CYA), yeast extract sucrose agar (YES) [Samson et al., 1995], and rice maize agar (RMA) [Bullerman, 1974], and incubated at 25°C for 7 and 14 days. After incubation, the tubes were first heated to kill the mold, which was then extracted with chloroform according to the method of Bullerman [1974]. Detection of toxins was performed under visible, short- and long-wave ultraviolet light on Thin Layer Chromatography (TLC) plates before and after spraying with H_2SO_4 and heating at 110°C [Scott and Kennedy, 1976].

Standards

Reference standards used in the study came from different origins. Roquefortine C was bought from CSIR Food Science and Technology (South Africa). PR toxin was a gift from Dr. S.

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TABLE I. Toxins Produced by Penicillium Strains Cultured on RMA, YES, and CYA at 25°C for 7 and 14 Days*

		7 Days			14 Days		
Strain	Identity	RMA	YES	CYA	RMA	YES	CYA
M 471	*P. crustosum*	A, C	A	C++	C	C++	C
M 512	*P. crustosum*	A	—	A, C++	C	C++	C+
M 514	*P. crustosum.*	—	—	—	—	—	—
M 515	*P. paneum* Frisvad	P	C	C	C	C	C++
M 516	*P. roqueforti* var. *roqueforti*	—	A++, P+	A	C−, B	P	A++, C++
M 518 (IBT 5214)	*P. roqueforti* var. *roqueforti*	A	—	A++, C++	A?, B?, C	A?, C+	C++
M 519 (IBT 5426)	*P. roqueforti* var. *roqueforti*	—	—	—	C	P	A, C
M 520 (IBT 5440)	*P. roqueforti* var. *roqueforti*	A, P	A, P, B++	A	A, C	P++, B++	AP++, C
M 521 (IBT 6884)	*P. roqueforti* var. *carneum*	A, C	A, C	A, C	A, C	AB+, C	A++, C
M 522 (IBT 10101)	*P. roqueforti* var. *roqueforti*	—	—	—	A+	A, P++	B
M 523 (IBT 10050)	*P. roqueforti* var. *roqueforti*	—	A, C	A, C	A+, C	A, P++	A++
M 188	*P. roqueforti* var. *roqueforti*	C?	A, B?, C	C	P, C	—	P++, C
M 189	*P. roqueforti* var. *roqueforti*	A, C	A, C	A, C	A+, B?, C	P++	A, C
M 491	*P. chrysogenum*	P	—	P	P++, C	—	A, C

*A, isofumigaclavine A; B, isofumigaclavine B; C, roquefortine C; P, PR toxin; —, no toxin detected. + and − are used for higher and lower concentrations of the toxin compared to ''normal level,'' and ? for dubious cases.

Moreau (INSERM U.42 Domaine du Certia, 59650 Villeneuve d'Ascq, France), and festuclavine and isofumigaclavine A and B were gifts from Dr. P. Scott (Food Research Division, Health and Welfare, Ottawa, Canada). Small quantities of standards were also isolated from our own experiments.

Wine Samples

A kit containing grape concentrate, yeast culture, and yeast nutrients (Willes 9-Days Wine from Vinland, Linköping, Sweden) was purchased from a local shop. The concentrate was diluted and divided into seven flasks, each containing 3 l of grape juice (must). All flasks were autoclaved at 110°C for 30 min before use. Three different experiments were performed.

In the first experiment, a quick fermentation process (to produce a wine in 9 days) was started. The penicillium strains chosen for inoculation of the grape juice (M 188 and M 512) were first grown on agar slants with malt extract agar (MEA) at 25°C for 7 days. Then the conidia from each strain were collected and suspended in 0.1% peptone in water. From this suspension, 25 ml were transferred to the wine samples. The conidia count in the suspension, confirmed by routine agar-plating on MEA, was log 7.8 (M 188) and log 7.2 (M 512) colony producing fungies (cfu) per ml. The inoculated samples were kept at room temperature (22°C). After 5 days, when the mold completely covered the surface of the samples, 1.4 g of wine yeast and 2.7 g of yeast nutrients were added to the samples. This first experiment also included a wine control with no mold added.

In a second experiment, a weaker fermentation was started 14 days after inoculation (with M 521 and M 522, respectively). At that time an indication of toxin production was found in one of the inoculated must samples. Only 0.5 g of yeast was added and no additional yeast nutrients were used. The same procedure as in the first experiment was used for conidia production and inoculation. The conidia count was this time log 7.6 (M 521) and log 6.6 (M 522) cfu per ml. Subsamples for toxin analyses were taken continuously during the 5-week yeast fermentation process.

A third experiment was performed to determine if inoculated conidia could grow and produce toxins in a wine ready to drink. The finished wine from the control in the first experiment was used. The clear phase of the wine was divided into two 500-ml samples. From each of the finished wine samples in the second experiment, conidia from the mold-cake on the surface of the samples were taken and suspended in 25 ml peptone-water and added to the respective 500-ml samples. The conidia count was log 3.4 (M 521) and log 5.2 (M 522) cfu per ml. Samples were observed daily for 3 weeks for any sign of mold growth.

All samples from the wine experiments were analyzed on TLC plates as described above, after extracting 50 ml of sample twice with 50 ml of chloroform. The combined chloroform layers were evaporated to dryness and dissolved in 100 µl chloroform before application on TLC plates.

Identification of Neurotoxins in Must and Wine by HPLC-DAD

A method for simultaneous detection of PR toxin, roquefortine C, and isofumigaclavines A and B on HPLC with Diode Array Detection was evaluated in parallel with the work above. The method has been used for confirmation of some positive samples in the wine experiments without any further purification than for the TLC method. The final samples were dissolved in methanol instead of chloroform.

In the HPLC method, the toxins were separated on a LiChroCART 250-4 Superspher 100 RP column from Merck (E. Merck, D-6100 Darmstadt, Germany) with a

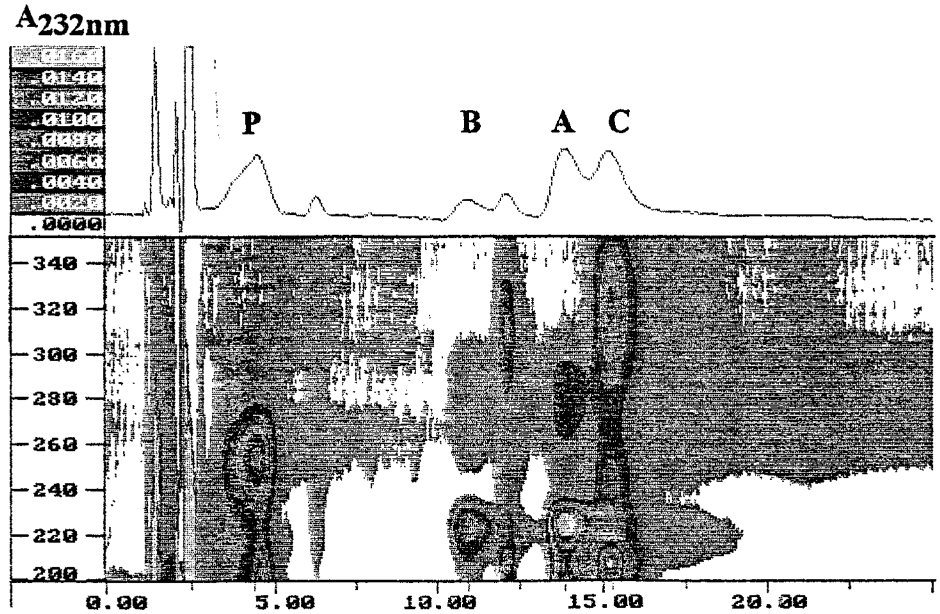

Fig. 1. HPLC-DAD chromatogram of the neurotoxins roquefortine C, PR toxin, and isofumigaclavines A and B. Retention times: P, PR toxin 4.4 min; B, isofumigaclavine B 10.9 min; A, isofumigaclavine A 13.9 min; C, roqueortine C 15.3 min; and festuclavin 22.6 min (not included in the chromatogram). Mobile phase: gradient of A, MeOH-H_2O (56 + 44); and of B, MeOH-10 mM $NH_4H_2PO_4$ in H_2O (56 + 44). Time program: 0–5 min, 10% B; 5–20 min, 10–50% B; 20–25 min, 50–10% B; 25–30 min, 10% B stop.

gradient of 10 mM ammonium phosphate and methanol at a flow rate of 1.0 ml/min. Column temperature was set at 55°C. Spectra were collected between 200–400 nm. Retention time and spectra of samples and corresponding standards were compared for identification. A chromatogram with retention times for the toxins is given in Figure 1, together with information on the ammonium phosphate gradient.

RESULTS AND DISCUSSION

Fourteen strains of the genus *Penicillium* were tested for their ability to produce roquefortine C, PR toxin, isofumigaclavines A and B, and festuclavine. Of nine tested strains of *P. roqueforti*, eight formed roquefortine C in at least one of the media used. Isofumigaclavine A and PR toxin were formed by nine and seven strains, respectively. Of interest is that the variety *P. roqueforti* var. *carneum* formed isofumigaclavine A, which was not known earlier [Frisvad and Filtenborg, 1989]. *P. paneum* Frisvad, which is a new species (Frisvad, personal communication), formed roquefortine C on all media. Two of three strains of *P. crustosum* were toxin producers and were able to form roquefortine C and isofumigaclavine A. The strain of *P. chrysogenum* formed PR toxin, roquefortine C, and isofumigaclavine A. Table I shows that all strains except one could form at least one of the toxins, when cultured on RMA or CYA media for 14 days, while on the YES medium another two strains did not form any toxin. When comparing toxin production from all strains after a culturing time on the chosen media for 7 and 14 days, respectively, it is obvious that the longer culturing time generally results in a higher toxin production. The yeast extract-containing media (CYA and YES) seem to give a higher toxin production than the RMA medium, with a few exceptions. It can also be noted that a toxin found after a culturing time of 7 days in some cases has disappeared or been replaced by another toxin after 14 days. When testing a mold strain for its ability to produce toxin, it seems to be necessary to use different media and culturing times to get optimal conditions for toxin production.

Three wine experiments were performed. In the first experiment, two flasks were inoculated with conidia from *P. roqueforti* var. *roqueforti* (M 188) and two other flasks with *P. crustosum* (M 512). Fermentation was started 5 days after inoculation, when the mold completely covered the surface of the samples. The instructions for a ''quick 9-day wine procedure'' were followed, causing a very strong fermentation process which stopped mold growth as well as toxin production. No toxin was found before, during, or after fermentation. An example from the analyses is shown at left on the TLC plate in Figure 2. The samples (control, M 188 #1, M 188 #2, M 512 #1, and M 512 #2) were analyzed 8 days after inoculation and 3 days after the fermentation process was started.

In the second experiment, a weaker fermentation was started in two other flasks (single experiments) 14 days after inoculation with *P. roqueforti* var. *roqueforti* (M 522) and *P. roqueforti* var. *carneum* (M 521), respectively. At that time, the surface of the samples was covered with mold and isofumigaclavine A was detected in one sample (M 522). The fermentation started quickly but not as vigorously as in

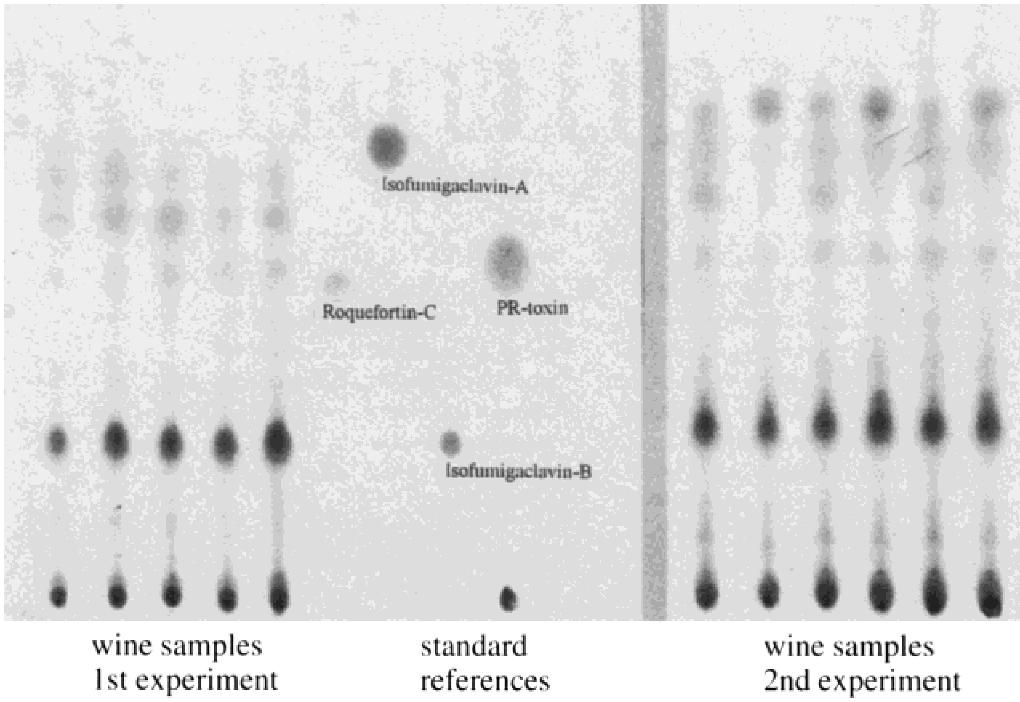

Fig. 2. TLC analyses of roquefortine C, PR toxin and isofumigaclavines A and B in wine samples on silica F 1500/LS 254 in chloroform-methanol-ammonia (90 + 10 + 1). (Contact corresponding author for color version of Fig. 2 if needed).

the first experiment. After another 7 days, isofumigaclavine A was detected also in the other sample (M 521). Continuous analyses showed a slow increment of isofumigaclavine A during the 5 weeks' fermentation process. Figure 2, right side, shows positive isofumigaclavine A spots for the samples of M 521 and M 522 analyzed 21, 22, and 23 days after inoculation and 7–9 days after fermentation was started. No other toxins were detected. The spots interfering with isofumigaclavine B in Figure 2 were separated from the standard in a TLC system of chloroform-methanol (9 + 1). When the fermentation process stopped, the alcohol content [analyzed by Gas Chromatography with Flame Ionisation Detection (GC-FID)] in the finished wine was 12%, but in spite of that, the mold conidia were still able to grow and form colonies on malt agar plates when tested. Confirmation of isofumigaclavine A in the finished samples was performed by other TLC systems and by HPLC-DAD, where retention times and spectra were identical for standards and samples. An approximate concentration of isofumigaclavine A in both final samples, after the cleaning steps, was about 0.1 mg/l.

In the last wine experiment, the wine control (containing 12% alcohol) from the first experiment was divided into two subsamples and inoculated with conidia taken from the two strains in the finished wine from the second experiment above. The samples were checked daily without any sign of mold growth. After 3 weeks, the samples were analyzed, but no toxins were found and the experiment was stopped.

This study has shown that isofumigaclavine A can be formed in wine during the fermentation process if there has been contamination with a toxin-forming penicillium strain before fermentation has started and if the fermentation is weak and slow. Infections by *P. roqueforti* and *P. crustosum* on fruits and berries are very common, and if such contaminated products are used as wine starters without proper disinfecting, there is a risk, under the circumstances mentioned above, that consumption of such contaminated wine may cause acute poisoning.

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